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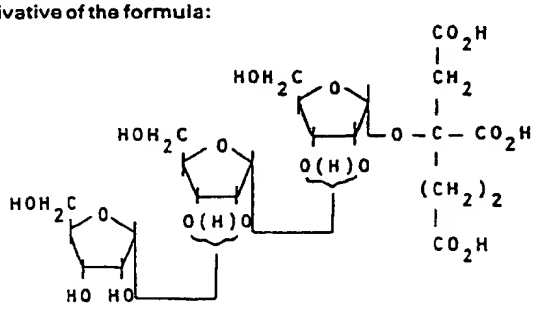
(54) Homocitric acid oligoriboside derivatives, process for their manufacture and their use as preventives of dental caries.

(57) The invention relates to a homocitric acid oligoriboside derivative of the formula:



Each glycosidic linkage between ribose moieties is ribosyl-(1→2)-ribosyl or ribosyl-(1→3)-ribosyl bond.

It may be obtained by cultivating a streptomyces, such as streptomyces SP MF 980-CF1 (FERM - P5430), and isolating it from the culture broth.

It is useful as a preventative for dental caries.

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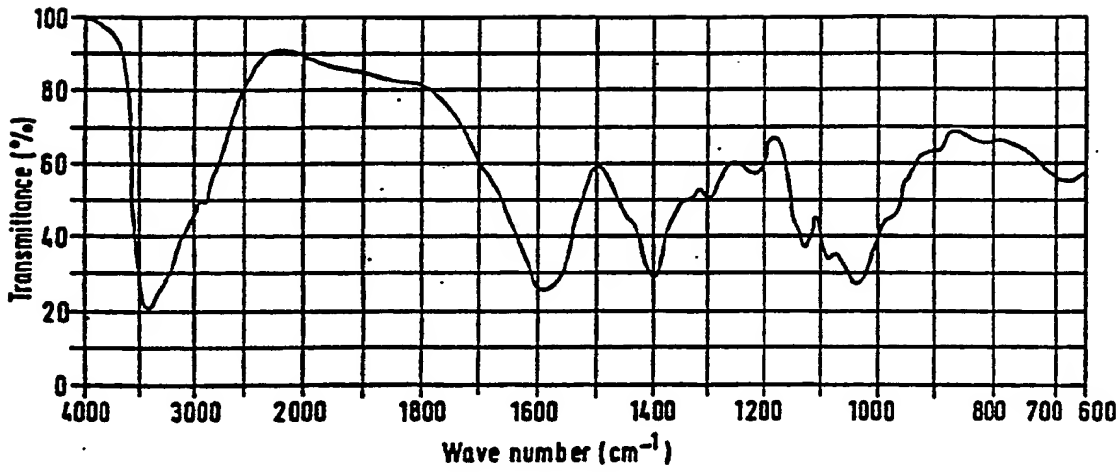
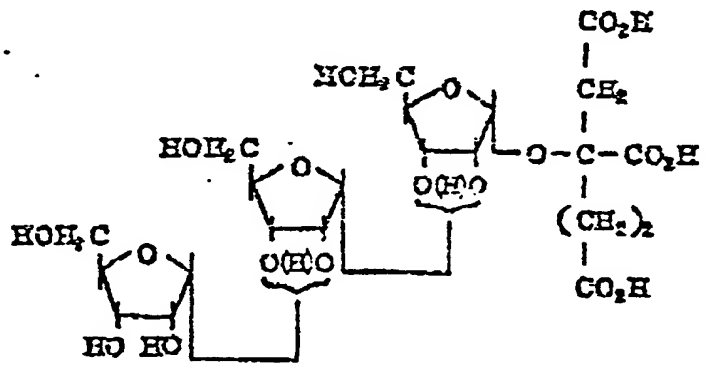
FIG. 3



BISEIBUTSU KAGAKU KENKYUKAI HOE 80/S 014 Dr. LI

Homocitric acid oligoriboside derivatives, process for their manufacture and their use as preventives of dental caries

The present invention relates to preventive of dental caries containing homocitric acid oligoriboside and its salts together with their manufacturing processes. The structure of the homocitric acid oligoriboside derivative is shown as follows.



Each glycosidic linkage between ribose moieties is ribosyl-(1→2)-ribosyl or ribosyl-(1→3)-ribosyl bond.

The homocitric acid oligoriboside described above is formed in the cultured broth of Streptomyces sp. such as Streptomyces sp. MF980-CF1 (FERM-P5430) and produced by the following method, for instance.

An organism, belonging to genus Streptomyces, capable of producing the homocitric acid oligoriboside in this invention is cultured in an usual medium consisting of carbon and nitrogen sources, inorganic ions and, if necessary, organic microelements, such as vitamins and/or amino acids. The homocitric acid oligoriboside fromed and accumulated in the culture broth are extracted, separated and collected. As the carbon source in the medium, carbohydrates such as glycerol, glucose, maltose, sucrose, lactose, starch and dextrin can be used for the production. Especially, maltose or potato starch is suitable. Soy bean meal, peanut meal, cotton seed meal, dry yeast, peptone, meat extract, casein, corn steep liquor, nitrate nitrogen and ammonia nitrogen etc. can be used as the nitrogen source. Cotton seed meal or corn steep liquor is especially preferable. If necessary, the medium is further added with magnesium, manganese, sodium, potassium, iron, calsium, chlorine, phosphate and/or sulfate ions. Aerobic cultivation by aeration, agitation or shaking and incubation temperature ranging from 25°C to 30°C are suitable for the production. Any other incubation temperature allowing the growth of the producer organism for the production of homocitric acid oligoriboside can be employed. Incubation of the culture is continued until the said substance is accumulated enough in the cultured broth. Usually, the incubation time is 2 to 7 days.

Streptomyces sp. MF980-CF1 (FERM-P5430) is one of the organisms having the ability to produce homocitric acid oligoriboside.

Microbiological properties of this strain is as follows. The strain was isolated from a soil collected around Lake Kawaguchi.

a) Morphology

Strain MF980-CF1 grown well on agar media shows linear or spiral structure of aerial mycelia elongated from simple-branched, well elongate substrate mycelia. When matured, spore chains of 10-50 cylindrical spores (0.8 x 1.8 µm) are formed on top of the mycelia. Observations by electron microscopy revealed smooth surface spores and no spiny or hairy structure on spores. Neither flagella or sporangia observed. Consequently, it can be classified in typical Streptomyces sp.

b) Characteristics on various media

1) Sucrose-nitrate agar (27°C incubation):

White (white a) and poor aerial mycelia are formed above the substrate growth of yellowish brown color (2 db extra pastel series for hue, by Color Harmony Manual). No distinctive diffusible pigments are formed in the medium.

2) Glycerol-Asparagine Agar (27°C incubation):

Substrate growth shows weak reddish brown (5 lg, hue 5) color. White aerial mycelia first appear on the peripheral parts of the colony and gradually cover the whole parts. No distinctive soluble pigments are formed.

3) Starch Agar (27°C incubation):

Borwnish white (5 ba, near gray series) aerial mycelia are formed above the orange (4 pe, hue 4) colored substrate growth. No distinctive soluble pigments are formed.

4) Tyrosine Agar (27°C incubation):

Substrate growth shows reddish brown (5 lg, hue 5) color. White (white a) aerial mycelia are fomred on the peripheral part of the colony. Melanoid pigment is formed.

5) Nutrient Agar (27°C incubation):

Growth is poor and shows no distinctive color. Aerial mycelium formation is also very poor.

6) Yeast-Malt Agar (27°C incubation):

Grayish white aerial mycelia are formed above the brown (chm 6 ng, hue 6) substrate growth and gradually turn to purplish gray (chm 5 dc near gray series). Brownish melanoid pigment is formed.

7) Oat Meal Agar (27°C incubation):

Brownish gray (chm 13 fe near gray series) aerial mycelia are formed above the reddish orange (chm 5 le hue 5) colored substrate growth. No distinctive soluble pigment is formed.

c) Physiological properties

1) Temperature range for growth is 14-33°C.

2) Starch is hydrolysed on Starch Agar.

3) Skin milk is peptonized but not coaggulated.

4) Melanoid pigment is formed in Tyrosine Agar, Yeast-Malt Agar and Peptone-Yeast-Iron Agar media.

5) Nitrate is reduced.

6) Gelatin is not liquefied.

d) Utilization of carbon sources (Pridham and Gottlieb medium)

D-Glucose, L-arabinose, sucrose, D-xylose, inositol,

mannitol, D-fructose and L-rhamnose are utilized, but raffinose is not.

The above properties show this strain typically belongs to genus Streptomyces and has characteristics such as formation of spiral structure on its aerial mycelia and formation of melanoid pigment.

Those properties resemble those of the following species, but there are some differences as follows.

1) Streptomyces griseoaurantiacus:
No mealnoid pigment formation and very poor utilization of sucrose are different from those of strain MF980-CF1.

2) Streptomyces resistomycificus:
No pigment formation, utilization of raffinose and pH dependence of pigment color of substrate growth differ from those of strain MF980-CF1.

3) Streptomyces diastochromogenes:
No pigment formation on Yeast-Malt agar medium and utilization of raffinose show the difference between the species and strain MF980-CF1.

4) Streptomyces galbus:
Pigment formation on glycerol-asparagine agar, starch agar and oatmeal agar and very poor utilization of sucrose and rhamnose differ from strain MF980-CF1.

5) Streptomyces neyagawaensis:
Pigment formation on glycerol-asparagine agar, starch agar and oatmeal agar and utilization of raffinose are different from those of strain MF980-CF1.

6) Streptomyces bottoropensis:

Pigment formation on glycerol-asparagine agar and utilization of raffinose differ strain MF980-CF1 from the species.

Since there is no known species showing the characteristics of this strain in the genus Streptomyces, the species of this strain is concluded as a new one.

We named this strain as Streptomyces sp. MF980-CF1 and deposited at Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry as accession No. FERM-P5430.

This strain is mutative artificially or spontaneously as well as other strains of Streptomyces sp. For example, ultraviolet rays, X-rays and chemical reagents may use as mutagens. All the mutants thus obtained and produced the homocitric acid oligoriboside are included in this invention.

The homocitric acid oligoriboside can be obtained from the fermented broth by the known methods, based on its acidic character above all, the basic anion exchangers are most favourable to adsorbe this material.

As the anion exchangers, for example, Amberlite CG400 or CG-4B (Rhom and Haas Co.), Diaion PA316 or WA30 (Mitsubishi Chemical Industries) or DEAE Sephadex (Pharmacia) and others of OH^-, Cl^- or HCO_3^- form or those mixture form are applicable.

After washing the adsorbent with water, the adsorbed homocitric acid oligoriboside is eluted with solution of sodium hydroxide, ammonium bicarbonate or mineral salts with good yield. Usually sodium chloride (0.1-1 M) or ammonium bicarbonate

(0.1-1 M) are employed.

As homocitric acid oligoriboside is not adsorbed to the cation exchanger practically, the cation exchangers are available to remove the basic impurities.

Homocitric acid oligoriboside derivative can be separated by the thin layer chromatography of cellulose (Avicel) developed with the mixed solvent composed of nPropanol:3 M ammonium hydroxide (55:45 Vol) (Rf: 0.65). Based on this character, the effective purification of this substance is attained by employing the cellulose column chromatography with similar or related solvents.

Salt type of homocitric acid oligoriboside depends on the used solvents, for example, to form free form, sodium salt, lithium salt and so on.

As homocitric acid oligoriboside inhibits strongly dextran-sucrose (EC 2.41.5), it can be used as medicine and others. Especially, for effective prevention of dental caries, it can be added to toothpaste solely or with other effective materials. Dental caries is the decay of teeth caused by demineralization of the enamel surface with organic acids produced by the bacteria adhered to tooth surface. The bacterial adherence to the smooth surface such as tooth was mediated by the sticky insoluble dextran produced from sucrose by dextran-sucrases of a certain group of oral Streptococcus. So, the inhibition of dextransucrase means the prevention of dental caries.

For the purpose of dental caries prevention, homocitric acid oligoriboside derivative can be employed as an additive not only to the toothpaste but to chewing gum, soft drinks, candy and the other food stuffs.

The detailed explanation will be described in the following illustrative procedures.

1) The production of homocitric acid oligoriboside. Streptomyces sp. MF980-CF1 (FERM-P5430) grown on the slant agar medium was inoculated to the medium composed of 1% maltose, 1% corn steep liquor, 1% Pharma media (Traders Oil Mill Company), (pH 6.2, 125 ml in 500 ml Sakaguchi flask). After the culture with shaking at 27°C for 5 days, the pH of the combined fermented broth (5 l) was adjusted to pH 8.0 with sodium hydroxide. Then the fermented broth was heat treated (60°C, 30 min) and filtered. The obtained filtrate (4.5 l) was passed through the column of Diaion PA316 (OH form, 1.3 l, Mitsubishi Chemical Industries Co.), and the column was washed with 10 l of water.

The adsorbed homocitric acid oligoriboside was eluted with 1 M ammonium bicarbonate. The combined active fractions (1 l, yield 83%) was concentrated to 80 ml under reduced pressure.

The concentrate was gel filtrated by Sephadex G-15 (1 l, Pharmacia Co.) with water as the eluting solvent. Each 50 ml-fraction was collected. The activity was found in No. 11-15 fractions. The combined active fraction (yield, 72%) was directly applied to the DEAE-Sephadex A-25 column (HCO_3^- form, 400 ml, Pharmacia Co.) and eluted with a linear gradient of 0.1-0.7 M ammonium bicarbonate. The activity was found in the

fractions about 0.25 M of ammonium bicarbonate. The active fractions were combined (yield 64%) and the ammonium bicarbonate was removed under reduced pressure. The concentrate thus obtained was applied successively to DEAE-Sephadex A-25 (Cl^- form, 200 ml, Pharmacia Co.) and eluted with a linear gradient of 0.01 M-0.5 M sodium chloride.

The activity (yield 58%) was found in the fractions about 0.17 M of sodium chloride. The active fractions were combined and concentrated to 1 ml, and desalted by a long column of Sephadex G-15 (30 ml) with water as eluting solvent (166 mg, yield 55%). After chromatography of silica gel (Silica AR CC-7, Mallinckrod Co.) with the mixed solvent (nPropanol:3 M ammonium hydroxide 70:30 Vol), the white crystals were obtained (90 mg, yield 49%).

2) Properties of the homocitric acid oligoriboside Na-salt.

1. White powder, mp 205-210°C (dec.).

2. Soluble in water; insoluble in organic solvents.

3. Hydrolysis of the homocitric acid oligoriboside gives riboses and homocitric acid.

4. Elemental analysis; Found: C 40.15, H 5.31%; Calcd. for $C_{22}H_{31}O_{19}Li_3 \cdot 2H_2O$: C 40.24, H 5.33%.

5. UV spectrum has no maximum.

6. Positive color reaction with phenol-sulfuric acid and orcinol-hydrochloride. Negative color reaction with ninhydrin, Nelson-Somogyi and diphenylamine-sulfuric acid.

7. The ^{13}C nmr spectrum in D_2O shows in Fig. 1.

8. The ^1H nmr spectrum in D_2O shows in Fig. 2.

9. The IR spectrum (KBr disk) shows in Fig. 3.

These facts indicate that the homocitric acid oligoriboside derivative in this invention has the structure described previously.

3) Inhibitory activity to dextransucrase.

Used dextransucrase was prepared as follows; Streptococcus mutans ATCC 27607 was inoculated to Brain Heart Infusion bouillon (Eiken Chemical Co.), and grown overnight at 37°C. The culture supernatant obtained by centrifugation was precipitated with ammonium sulfate at 50% saturation. The precipitated enzyme was desalted and purified with common procedures.

Inhibitory activity to dextransucrase was assayed as follows; 2.7 ml of substrate solution (0.3% sucrose, 0.04% sodium azide, 30 mM potassium chloride, 30 mM sodium chloride in 50 mM imidazole-HCl buffer (pH 6.8), 0.3 ml of test solution or water as control, and 50 µl of dextransucrase were mixed.

After incubation at 37°C for 14 hrs, the turbidity at 600 nm was measured against water as blank, and the percent inhibition was calculated from the following equation:

$$\text{Percent inhibition} = \frac{(\text{Turbidity of control}) - (\text{Turbidity of test})}{(\text{Turbidity of control})} \times 100$$

The definition of 1 unit of this material is the amount which gives 50% inhibition.

Fig. 4 shows the relation between the concentration of

homocitric acid oligoriboside and the percent inhibition determined by the assay method described above.

4) Inhibition of Streptococcus mutans adherence.

Heart Infusion bouillon (Difco) containing various concentrations of sucrose was autoclaved and added aseptically with various amounts of homocitric acid oligoriboside aqueous solution (filter-sterilized) or sterile water for control (Final volume is 3.0 ml and final concentrations were shown in Table 1). Each tube was inoculated $1x10^5$ CFU of Streptococcus mutans ATCC 27607 grown overnight at 37°C in Brain Heart Infusion bouillon (Difco) and incubated anaerobically in a candle jar at 37°C for 14 hr. The tube was positioned at an angle 30° from the horizontal. Nonadherent cells were removed by pouring off the broth and the test tube was washed three times with 3 ml of 50 mM phosphate buffer (pH 6.0). The adhered plaque (insoluble dextran and cellular aggregates formed as a result of insoluble dextran production) was suspended in 3 ml of phosphate buffer described above by mechanical stirring and by scraping the surface with a glass rod.

Turbidity of the suspension was measured at 600 nm. The results were shown in Table 1.

5) Explanations of figures.

Fig. 1 shows the ^{13}C nmr spectrum of homocitric acid oligoriboside Na-salt in this invention.

Fig. 2 shows the ^{1}H nmr spectrum of homocitric acid oligoriboside Na-salt in this invention.

Fig. 3 shows the IR spectrum of homocitric acid oligoriboside

Na-salt in this invention.

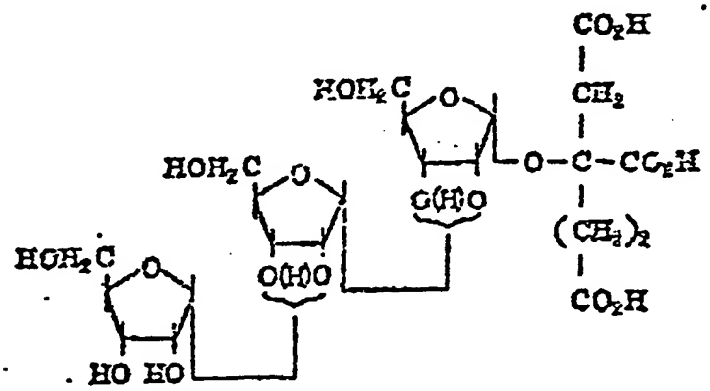
Fig. 4 shows the relationship between concentration of the homocitric acid oligoriboside Na-salt and inhibition ratio (%) to dextransucrase activity.

Table 1. Inhibition of Streptococcus mutans Adherence

Concentration (µg/ml) of Homocitric acid oligoriboside derivative (sodium salt)	Concentration (%) of sucrose					
	0.5		1.0		2.0	
	Turbidity (600 nm)	Percent inhibition	Turbidity (600 nm)	Percent inhibition	Turbidity (600 nm)	Percent inhibition
0	0.617	—	0.595	—	0.502	—
50	0.510	17	0.340	43	0.333	33
100	0.249	60	0.173	71	0.132	73

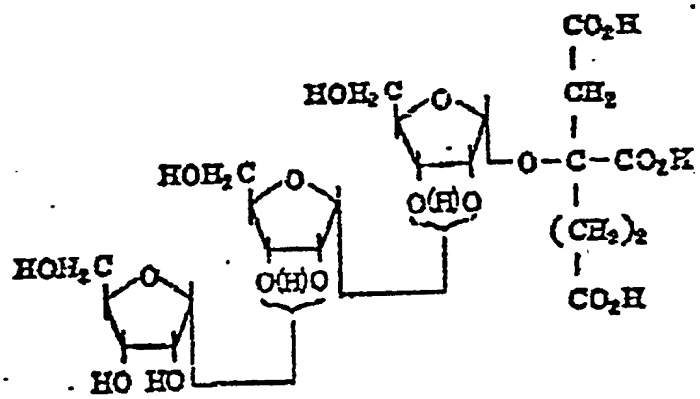
Patent claims:

(1) Homocitric acid oligoriboside derivative of which structure is shown below and its salts.



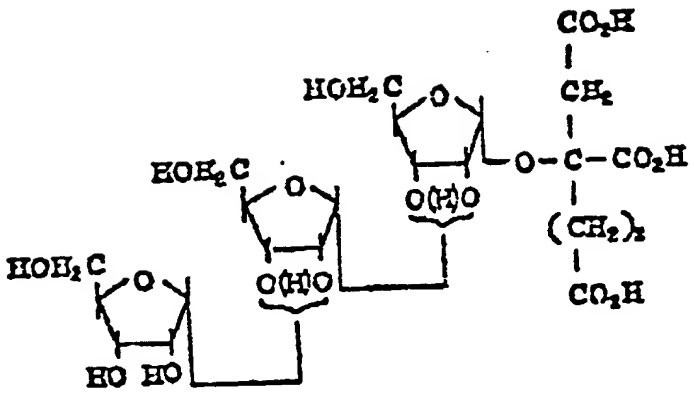
Each glycosidic linkage between ribose moieties is ribosyl-(1→2)-ribosyl or ribosyl-(1→3)-ribosyl bond.

(2) Processes obtaining homocitric acid oligoriboside derivative (the structure shown as follows) which is isolated from cultured broth of Streptomyces sp.



Each glycosidic linkage between ribose moieties is ribosyl-(1→2)-ribosyl or ribosyl-(1→3)-ribosyl bond.

(3) Preventives of dental caries containing homocitric acid oligoriboside derivative shown as follows and its salts.



Each glycosidic linkage between ribose moieties is ribosyl-(1→2)-ribosyl or ribosyl-(1→3)-ribosyl bond.

(4) Detailed explanations of the invention

FIG. 1

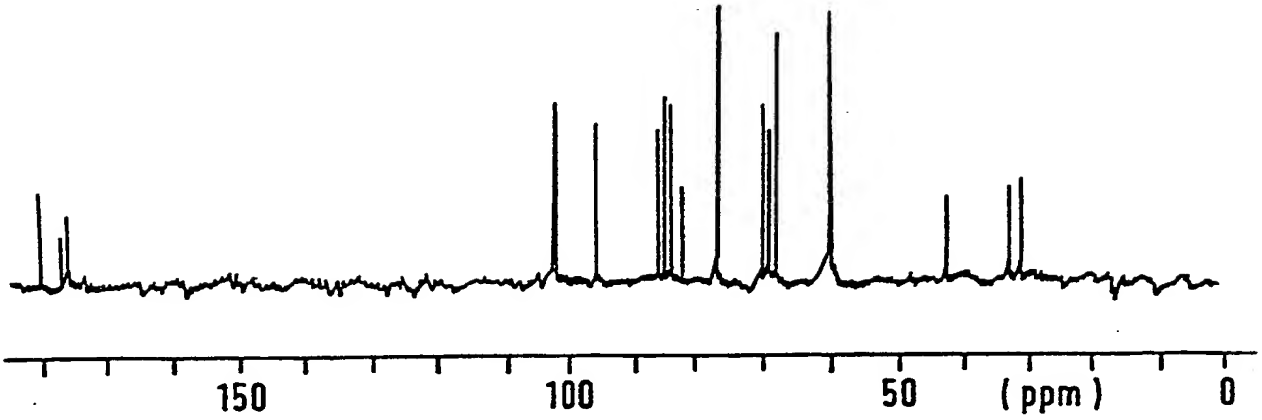


FIG. 2

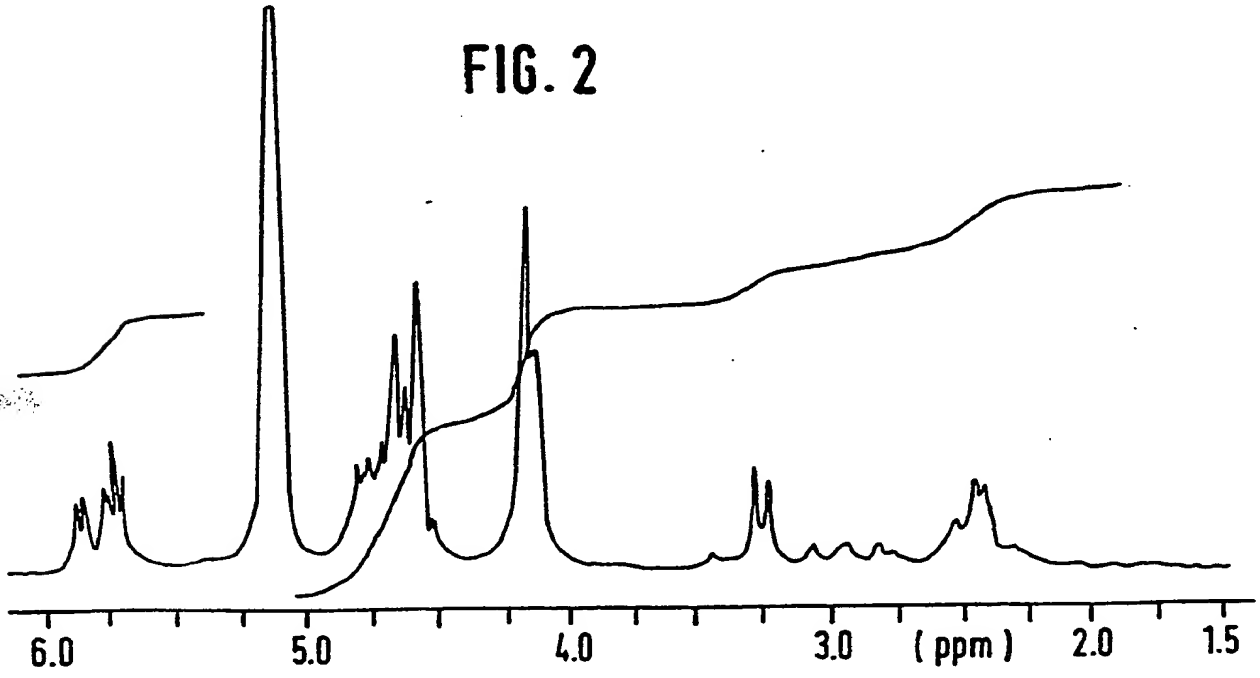


FIG. 3

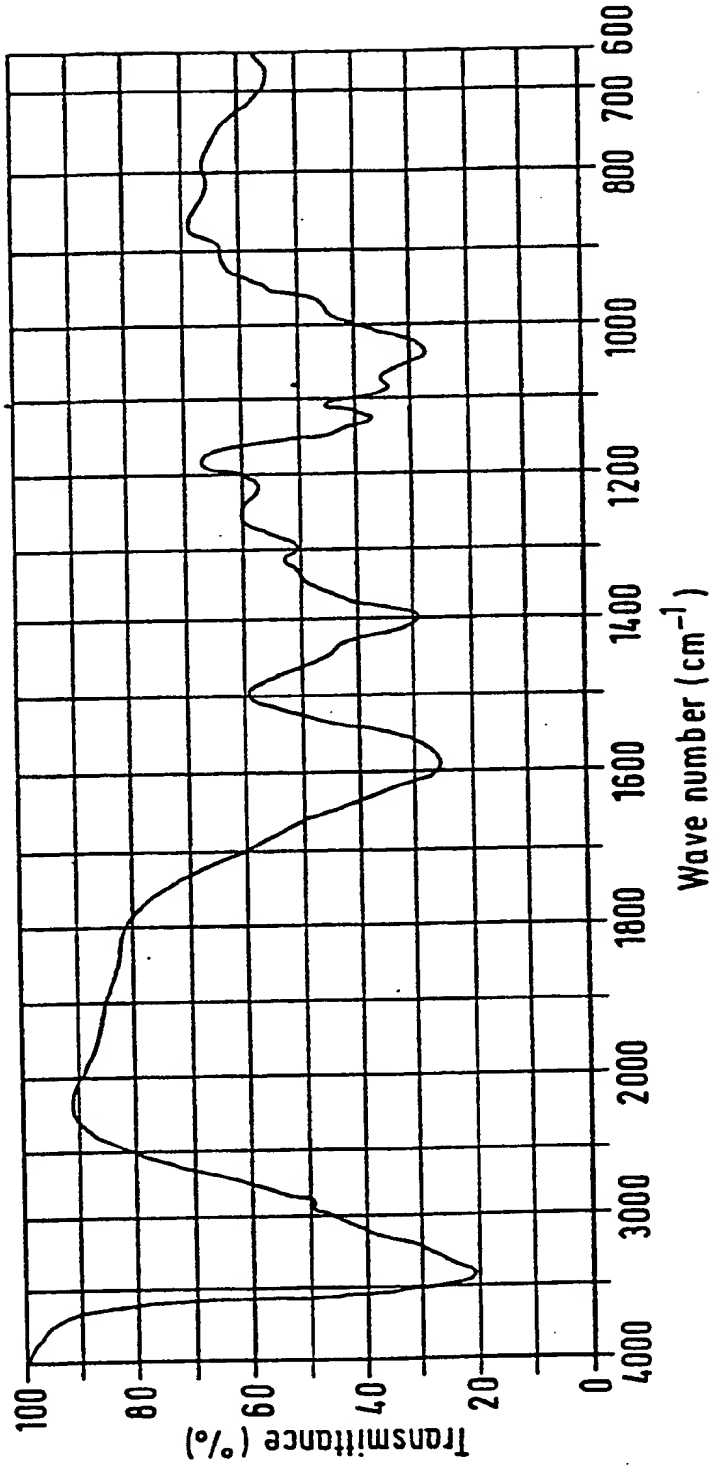
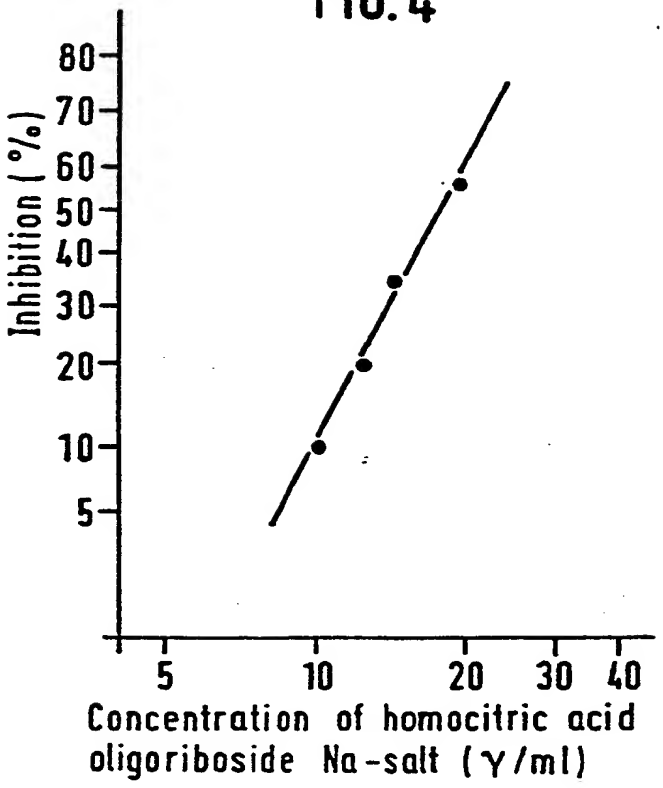


FIG. 4



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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number: EP 81 10 1534

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
	No relevant documents have been disclosed.	

CLASSIFICATION OF THE APPLICATION (Int. Cl.³)

C 07 H 15/04
C 12 P 19/44 //
(C 12 P 19/45
C 12 R 1/465)
A 61 K 7/24

TECHNICAL FIELDS SEARCHED (Int. Cl.³)

C 07 H 15/04
C 12 P 19/44
C 12 R 1/465

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 1-3
Claims searched incompletely: 4
Claims not searched:
Reason for the limitation of the search:

CATEGORY OF CITED DOCUMENTS

X: particularly relevant
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E: conflicting application
D: document cited in the application
L: citation for other reasons

&: member of the same patent family, corresponding document

Place of search	Date of completion of the search	Examiner
The Hague	04-06-1981	VERHULST

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